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The Role of Trace Elements in Gallstone Formation

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Introduction

As gallstone disease is one of the most common illness in the surgical and medical field, the pathogenesis or etiology of gallstones, though extensively studied, has not yet been elucidated[13]. The analysis of gallstones also has been actively undertaken using various techniques and instruments because the precise identification of their components is one of the first steps in clarifying the mechanism of their formation.

However, there are various difficulties and some confusion in the classification of the gallstones since they are composed of both different and similar components. For example, there are two kinds of colored stones other than the so-called cholesterol stone in Japan; bilirubin stone and black stone as described here. The former is sometimes called 'bilirubin calcium stone' or 'calcium bilirubinate stone'[18], and the latter is called 'pure pigment stone' or 'black pigment stone'[10,32,40]. These two stones are frequently confused because their appearances are similar, but they differ in many respects such as the degree of blackness, the structure of their cut surface, their location in the biliary tracts and their compositions[16,21].

Among gallstone patients in western Japan, those with cholesterol stones comprised 67%–74%, bilirubin stone 20%–27%, and black stone 6%; unlike in the U.S.A. or European countries where a high incidence of black stones and a low incidence of bilirubin stones are characteristic. This may be the chief reason for the confusion of black stones and bilirubin stones among these countries, as both of them are called pigment stones. Though both cholesterol stones and black stones mostly originate in gallbladder, bilirubin stones are found in all regions of the biliary tract including the intrahepatic duct. The percentage of bilirubin stones in the gallbladder was reported to be 47%, in the choledochus 29% and in both of these regions 24%[16].

Trace elements such as copper and manganese are specifically related to the bile via which they are excreted into feces, as described previously[26]. Though these elements have been found in various gallstones using infrared spectroscopy or X-ray microanalyzer[4,5,32], their precise quantification in gallstones is difficult because of inadequate analytical techniques.

In this work, a method was established for the determination of trace elements using flameless atomic absorption spectrometry; simultaneously, the major components such as cholesterol,

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bilirubin, palmitate and calcium were determined in the representative gallstones. In addition to identifing the role of trace elements, an attempt was made to grasp the general configuration of gallstones mathematically, using multivariate analysis, factor analysis or principal component analysis, for the multiple data.

Based on these analyses, the most important constituents in human gallstones were determined.

Materials and Methods

Sample Collection

More than 200 gallstones extirpated operatively at Kyoto University Hospital were roughly classified into cholesterol stones, bilirubin stones, black stones, and others; the most typical stones representative of the three groups were chosen by the macroscopic morphology. Cholesterol stones are white to light yellow and have a relatively smooth surface; the radial cross-sectional pattern sometimes reveals a small dark center. Bilirubin stones appear light to dark brown or black on the surface, and on cross section, have multiple concentric layers which are brown or dark brown. Black stones are dark brown or black on the surface, and on cross section, have a smooth conchidal appearance resembling the surface[16,21].

Both cholesterol stones and black stones in the gallbladder were selected to eliminate any differences due to their location. On the other hand, bilirubin stones in both the gallbladder and choledochus, were investigated because they are frequently found in both regions.

Intrahepatic stones are usually considered as bilirubin stones, and the incidence is low. However, these stones were also analysed because of the clinical importance of this disease and the fact that these stones are difficult to manage; frequently polysurgery is required[22].

Table 1 summarized the type of gallstone, number and location with respect to the patients included in this study.

Gallstone Analysis

Gallstones were stocked in a desiccator after washing with pure water and pulverized with an agate mortar; this stone powder was used for the determination of the various components.

Trace elements and other metals: 30–300 mg of stone powder in a borosilicate flask was dissolved with concentrated HNO_3 and $HClO_4$ on a hot plate for four hours by the wet digestion method, then pure water was added to make up 50 ml. Copper, manganese and zinc were determined by flameless atomic absorption spectrometry (Shimadzu AA 640-13 model, GFA 2 model, Kyoto, Japan) equipped with an autoinjector (Shimadzu AIU 1 model). The operating parameters of these instruments were the same as those previously described[27]. To eliminate error due to contamination during this procedure, all reagents were carefully chosen. Pure water was the water refined by an infrared, non-boiling type distillation apparatus (Daiken Quartz Glass, Tokyo, Japan). The reproducibility of this technique and the recovery test were very satisfactory. In evaluating the same stone powder five times, the coefficients of variation were under 5% for every elements; the recovery of three elements was 95 to 103% in four stones.

Table 1. Gallstones Investigated in This Study.

Type of Gallstone	Number	Location	Patients		
			Female	Male	Age
Cholesterol Stones	18	Gallbladder	9	9	13–65
Bilirubin Stones	6	Gallbladder	3	3	50–76
	18	Choledochus	6	12	42–74
Black Stones	18	Gallbladder	13	5	18–76
Intrahepatic Stones	24	Intrahepatic duct	15	9	32–82

Simultaneously, calcium, magnesium and other metals were determined in the solutions of gallstone powder by flame atomic absorption spectrometry. Phosphorus was determined with the method of FISKES and SUBBAROW[14].

Organic components: Cholesterol, bilirubin and palmitate were evaluated using the extraction method of MUKAIHARA[20]. In this procedure, 10 mg of the gallstone powder was first dissolved in 10 ml of petroleum ether. After aspiration of the supernatant solution, 10 ml of 1 N-HCl was added and the mixture was immersed in an ultrasonic bath. Then, after aspiration of the supernatant, petroleum ether was again added. Finally, the petroleum ether was aspirated and 10 ml of dimethyl sulfoxide (DMSO) was added. The combined solutions of petroleum ether were used for the detection of cholesterol and palmitate, and the fraction of DMSO for bilirubin. However, a black residue remained after the subsequent extraction procedures in some gallstones. The volume of this residue was also measured.

Statistical Analysis

Student T test was carried out to identify the differences of various components among three stone types and further, correlation analysis, principal component analysis and factor analysis were performed using a computer (Hewlette Packard, System 45 B, U.S.A.)[23]. These multivariate analyses were carried out for the management of multiple data and their interpretation.

Results

I. General Configuration of Gallstones

Gallstone Components

Table 2 summarizes the analytical data.

Trace elements: Among all stone types, the amount of copper, manganese and zinc was widely distributed (Fig. 1). The cholesterol stones contained from 1.3 μg/g to 259 μg/g of copper, 0.4 to 140μg/g of manganese, and 0.1 to 81.3μg/g of zinc. The bilirubin stones in the choledochus contained 169 to 8,860μg/g Cu, 6.3 to 192μg/g Mn and 3.1 to 198μg/g Zn. Further, black stones contained 109 to 17,800 μg/g Cu, 608 to 5,440 μg/g Mn and 15.9 to 1,150 μg/g Zn. Generally, these trace elements were mostly found in black stones and to a lesser extent in cholesterol stones. Especially, a greater amount of manganese was found in black stones than in the other stones ($P<0.001$). The amount of copper and zinc, though not as remarkable as manganese, was

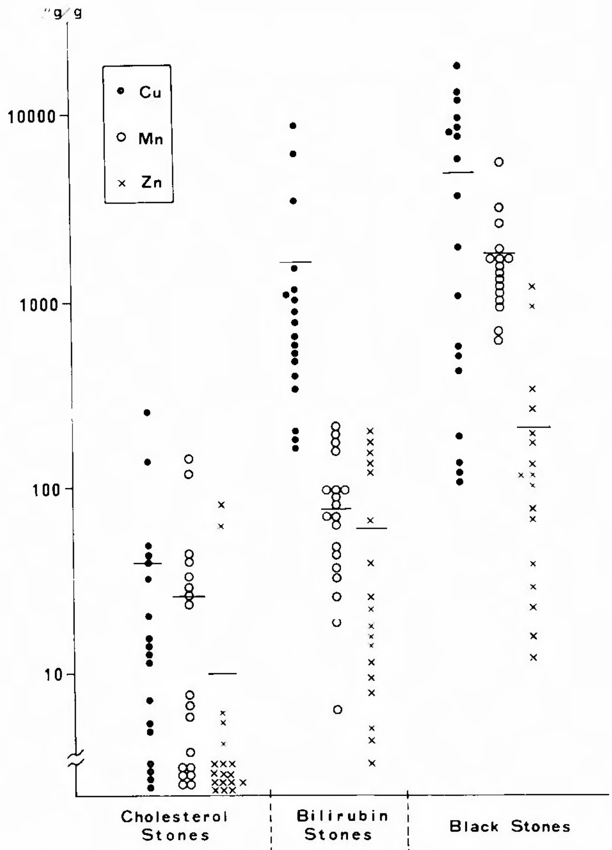

Fig. 1. The concentrations of trace elements in various gallstones.

significantly different between black stones and bilirubin stones ($P<0.05$). Comparing the three elements, there was more copper than manganese in all of the bilirubin stones, 11 of 18 cholesterol stones and 9 of 18 black stones. The amount of zinc was generally lower than the other two elements. Between bilirubin stones in the choledochus and those in the gallbladder, no significant difference was seen among the three elements.

Other major components: The percent of cholesterol in the three stone types was: more than 90% in cholesterol stones, 1.1% to 11.8% and 3.8% to 7.5% in bilirubin stones in the choledochus and gallbladder respectively, and 0.2% to 9.5% in black stones. Bilirubin was 0.1% to 2.7% in cholesterol stones, 20.2% to 38.7% and 22.0% to 30.8% in bilirubin stones in the choledochus and gallbladder respectively, and 1.4% to 18.8% in black stones. Palmitate in cholesterol stones was 0% to 7.3%, in bilirubin stones of the choledochus and gallbladder 4.8% to 24.2% and 15.1% to 39.5% respectively, and in black stones 0% to 3.7%. The percent of residue rarely reached 1.1% in cholesterol stones, however, it was 0.3% to 15.6% and 0.3% to 4.2% in bilirubin stones of the choledochus and gallbladder respectively, and in black stones 0.1% to 55.6%.

Interestingly, the proportion of bilirubin in bilirubin stones was remarkably lower than that of cholesterol in cholesterol stones, although they were the respective major components in each stone type. The amount of cholesterol in bilirubin stones was significantly higher than in black stones ($P<0.001$). On the other hand, bilirubin in black stones was remarkably greater than in cholesterol stones ($P<0.001$). Bilirubin stones contained a considerable amount of palmitate, and more of this salt component was present in bilirubin stones in the gallbladder than in those in the choledochus ($P<0.001$). There was no significant difference between cholesterol stones and black stones. Some residue was present in both bilirubin and black stones, but very little was seen in cholesterol stones; the amount of the residue in black stones was significantly higher than in bilirubin stones ($P<0.005$).

Excluding palmitate, there was no significant difference in cholesterol, bilirubin and the residue between the bilirubin stones in the choledochus and those in the gallbladder.

Other major ions: Calcium was the most dominant ion in all gallstones. Especially in black stones, a large amount from 2.8% to 43.3% was found. In bilirubin stones of the choledochus and gallbladder, it was 1.9% to 4.0% and 3.0% to 5.7% respectively. Between these two sites, there was a significant difference ($P<0.005$). Calcium in cholesterol stones was 0.1% to 3.9%.

Black stones contained the greatest amount of phosphorus and magnesium as well as calcium. Phosphorus was 1,700 to 8,420 $\mu g/g$ in black stones, 95.8 to 1,360 $\mu g/g$ and 76 to 1,510 $\mu g/g$ in bilirubin stones of the choledochus and gallbladder respectively, and 24.9 to 990 $\mu g/g$ in cholesterol stones. Magnesium was 1,930 to 7,280 $\mu g/g$ in black stones, 92.2 to 623 $\mu g/g$ and 252 to 428 $\mu g/g$ in bilirubin stones of the choledochus and gallbladder respectively, and 15 to 412 $\mu g/g$ in cholesterol stones.

Both calcium and magnesium were present to a far greater extent in black stones than in bilirubin stones; the amounts in bilirubin stones were greater than in cholesterol stones ($P<0.001$). Though the amount of phosphorus was high in black stones, there was no significant difference between bilirubin and cholesterol stones.

To summarize these results, cholesterol stones were mostly composed of a large amount of cholesterol and the proportions of the other components were low. Calcium, the residue and bilirubin comprised 50% of the weight of black stones. In bilirubin stones, bilirubin and calcium were the major components, but other organic substances such as palmitate, cholesterol or the residue were also present in considerable amounts. Bilirubin stones of the gallbladder contained a higher amount of palmitate and calcium than those of the choledochus. However, about 30% of the bilirubin and black stones consisted of unknown or undetected substances, which may be inorganic substances such as carbonate or organic substances such as bile acids, protein and/or low molecular bile pigment polymers[4,21,28,31,40].

The greatest amount of trace elements as well as other inorganic ions was found in black stones; a relative highly amount of copper was also present in bilirubin stones.

Statistical Analysis of Gallstone Components

Correlation analysis: Correlation coefficients among the 10 components described above were calculated in each type: cholesterol stone, bilirubin stone of choledochus and black stone, and in all of these stones (Table 3, a–d). However, the residue in cholesterol stones was excluded

Table 2. The Amount of Each Components in Various Gallstones

Gallstones	Location	Cholesterol (%)	Bilirubin (%)	Palmitate (%)	Ca (%)	P (μg/g)	Mg (μg/g)	Cu (μg/g)	Mn (μg/g)	Zn (μg/g)	Residue (%)
Cholesterol Stones (n=18)	Gallbladder	97.9±0.9	0.4±0.1	1.1±0.5	0.9±0.2	323±74.9	90±24	40.5±15.1	26.4±9.0	10.1±5.6	0.1±0.1
Bilirubin Stones (n=24)	Choledochus (n=18)	7.2±0.8	29.7±1.4	13.1±1.5	3.2±0.1	528±84.0	375±37	1620±557	78.7±13.2	59±16.8	4.1±1.0
	Gallbladder (n=6)	5.4±0.6	24.4±1.4	26.3±3.9	4.3±0.4	542±255	314±30	1120±212	83.4±34.5	37.7±16.0	2.1±0.8
Black Stones (n=18)	Gallbladder	0.9±0.5	8.7±1.3	1.3±0.3	19.1±2.9	30400±6040	3100±279	4930±306	1820±280	227±76.9	17.6±4.1
Intrahepatic Stones (n=24)	Intrahepatic Duct	21.8±4.1	25.8±2.5	17.4±3.7	3.1±0.8	241±61.5	308±37.1	1260±242	24.8±7.1	25.6±5.0	4.7±1.0

(Mean±S.E.)

Table 3. a) The Correlation Coefficients among Various Components in Cholesterol Stones.

Component	Cholesterol	Bilirubin	Palmitate	Ca	P	Mg	Cu	Mn	Zn
Cholesterol	1.000	−.293	.060	−.420	.243	−.154	−.193	.061	−.169
Bilirubin		1.000	.284	−.200	−.127	−.093	$\boxed{.930}$	−.117	$\boxed{.858}$
Palmitate			1.000	−.214	−.095	−.098	.408	−.138	.253
Ca				1.000	.190	.666	−.203	.470	−.153
P					1.000	.470	.055	$\boxed{.726}$	.160
Mg						1.000	−.106	$\boxed{.848}$	−.099
Cu							1.000	−.040	$\boxed{.939}$
Mn								1.000	−.054
Zn									1.000

$\boxed{}$: P<0.001, n=18

Table 3. b) The Correlation Coefficients among Various Components in Bilirubin Stones.

Component	Cholesterol	Bilirubin	Palmitate	Ca	P	Mg	Cu	Mn	Zn	Residue
Cholesterol	1.000	−.644	−.328	−.015	.333	−.048	−.213	−.195	.077	.019
Bilirubin		1.000	−.313	−.092	.143	.152	−.084	.403	.057	−.394
Palmitate			1.000	.372	−.138	−.331	−.229	−.137	−.215	−.185
Ca				1.000	−.185	−.432	−.509	.125	−.312	−.285
P					1.000	.049	−.248	.275	.281	−.333
Mg						1.000	.403	.097	−.189	.155
Cu							1.000	.035	.062	.493
Mn								1.000	.442	−.315
Zn									1.000	.112
Residue										1.000

□: $P<0.005$, $n=18$

Table 3. c) The Correlation Coefficients among Various Components in Black Stones.

Component	Cholesterol	Bilirubin	Palmitate	Ca	P	Mg	Cu	Mn	Zn	Residue
Cholesterol	1.000	−.293	.154	.196	−.210	−.194	−.311	−.289	−.182	−.323
Bilirubin		1.000	.349	−.721	.224	−.020	.522	−.084	.329	.458
Palmitate			1.000	−.201	.127	−.207	.038	−.068	.285	−.048
Ca				1.000	−.152	.120	−.695	−.063	−.496	−.805
P					1.000	.331	−.252	.745	.246	−.132
Mg						1.000	−.249	.238	.023	−.202
Cu							1.000	−.298	.435	.678
Mn								1.000	.372	−.161
Zn									1.000	.260
Residue										1.000

□: $P<0.001$, $n=18$

Table 3. d) The Correlation Coefficients among Various Components in Various Gallstones.

Component	Cholesterol	Bilirubin	Palmitate	Ca	P	Mg	Cu	Mn	Zn	Residue
Cholesterol	1.000	−.646	−.381	−.484	−.391	−.556	−.423	−.449	−.328	−.448
Bilirubin		1.000	.736	−.199	−.122	−.135	.121	−.171	.055	.046
Palmitate			1.000	−.251	−.275	−.326	−.118	−.310	−.105	−.166
Ca				1.000	.459	.715	.037	.566	.061	.063
P					1.000	.729	.210	.879	.464	.347
Mg						1.000	.382	.764	.408	.478
Cu							1.000	.249	.536	.758
Mn								1.000	.551	.391
Zn									1.000	.455
Residue										1.000

□: $P<0.001$, $n=54$

because it was only rarely detected in this group.

In cholesterol stones, there were significant relationships among copper, zinc and bilirubin, whereas manganese was highly correlated to phosphorus and magnesium. In black stones, copper was significantly correlated to the residue but inversely related to calcium. Moreover, manganese was highly correlated to phosphorus in black stones as well as in cholesterol stones. Unlike these two stone types, the composition of bilirubin stones was very complicated; the correlation coefficients of various components were relatively low, however, in bilirubin stones, as well as in black stones, copper was correlated to the residue and inversely related to calcium. Manganese and zinc were not significant correlated to other components. In these stones, the close relationship between bilirubin and calcium was noted[18], but no relation was seen from gallstone analysis. In all 54 gallstones, copper was highly correlated to the residue and manganese, to phosphorus and magnesium (Table 3-d).

Thus, from the results of correlation analysis, a significant relationship between copper and the residue, and between manganese and phosphorus were found. These findings are very important in identifying copper or maganese binding substances in gallstones and their role in gallstone formation.

Multivariate analysis: In order to classify the gallstones by their components and to define more precisely the constituents of the three types, factor analysis for exploratory use[23] was undertaken from the 540 items: 10 components each of 18 cholesterol stones, 18 bilirubin stones of the choledochus and 18 black stones. The fundamental concept of this analysis is that the correlation among multiple variables is dependent on the least number of 'Factors' contained commonly and potentially in each variable. Therefore, it is of most importance to define the common 'Factors' and estimate the weights of them to each variable. Although there are various combinations of procedures to obtain the terminal solution, R factoring for the preparation of the correlation matrix, principal component solution for the extraction of initial factors and orthogonal rotation were carried out. The correlation among 10 variables (gallstone components) and the solution of principal component analysis are shown in Table 3-d, and Table 4, respectively. The eigen value with each 'Component' indicates the amount of total variance accounted for by the 'Component' and is ordinarily selected as greater than 1.0. Cummulative percentages imply the proportion of total variance accounted for by several 'Components'. For example, it was 65.9% with two 'Components' and 80.9% with three 'Components'. Using this procedure, three significant common 'Factors' remained and were used for further analysis.

The terminal solution after the orthogonal rotation using the varimax method is shown in Table 5. The factor matrix represents regression coefficients of 'Factors' to one variable (one gallstone component). The importance of a given Factor for a given variable can be precisely expressed in terms of the variance in variable that can be accounted for by the Factor. For example, the variance of variable 1 (cholesterol) accounted for by the variation in Factor 1 was $(-0.555)^2$. Therefore, 30.8% of the variance of variable 1 is accounted for by Factor 1. Likewise, the variance of a variable accounted for by all Factors is given by the sum of the squares of the respective factor matrices. The total variance of a variable accounted for by the combi-

Table 4. Principal Component Analysis of Gallstone Components.

Eigen Vector		1	2	3
1.	Cholesterol	−.309	−.437	.256
2.	Bilirubin	−.024	.614	−.160
3.	Palmitate	−.143	.537	−.238
4.	Ca	.295	−.161	−.478
5.	P	.394	−.117	−.158
6.	Mg	.434	−.087	−.161
7.	Cu	.278	.212	.507
8.	Mn	.424	−.126	−.150
9.	Zn	.310	.105	.311
10.	Residue	.316	.156	.444
	Eigen values	4.294	2.297	1.501
	Cummulative percentage	42.9	65.9	80.9(%)

nation of all common Factors, is usually referred to as the communality of the variable. Thus, the nearer the values of communalities are to 1.0, the larger is the proportion of the variance of a variable accounted for by common Factors. For example, the proportion of variable 1, accounted for by only Factor 1, was 30.8% and by three Factors was 94.8%.

In this way, according to factor analysis, 10 components of gallstones were reduced to three significant 'Factors', and the weight of these three Factors to raw data were expressed statistically. Furthermore, the calculation of the Factor score of each gallstone can be plotted graphically in less dimensional space, in this case, three from 10-dimensional space.

Graphical Presentation

Gallstone classification: Based on their Factor scores, 54 gallstones were depicted in a three-dimensional space consisting of Factor 1 (F1), Factor 2 (F2) and Factor 3 (F3) as shown in Fig. 2. The axes of the three Factors are independent and orthogonal. The two-dimensional representation of F1 and F2 is shown in panel A of Fig. 2 and that of F1 and F3 in panel B of Fig. 2.

Table 5. Varimax Rotated Factor Matrix.

Variable		Communalities	Factor 1	Factor 2	Factor 3
1.	Cholesterol	.948	−.555	−.724*	−.340
2.	Bilirubin	.906	−.121	.939*	.099
3.	Palmitate	.836	−.244	.867*	−.154
4.	Ca	.775	.867*	−.024	−.149
5.	P	.737	.802*	−.105	.287
6.	Mg	.864	.861*	−.062	.345
7.	Cu	.821	.053	.071	.902*
8.	Mn	.841	.848*	−.121	.326
9.	Zn	.582	.273	.005	.712*
10.	Residue	.782	.018	.018	.866*

*: most weighed Factor.

From principal component analysis, the cummulative percentage of the combination of F1 and F2 was 65.9% and with the addition of F3 it became 80.9%. Thus, Fig. 2 shows a large part of the inputed data in spite of a small loss due to data manipulation.

By combining the two figures (panels A and B) with the aid of the sketch in the center, the position of each gallstone can be three-dimensionally imaged. In this figure, the cholesterol stone occupies the left-front-bottom region, the bilirubin stone the left-back region, and the black stone group in the right region. From this figure, gallstones can be classified into three groups according to macroscopic classification. This figure provides evidence for this classification.

Furthermore, this figure provides information not only of the gallstone classification but also of the role of the gallstone components. While each components weighed to each Factor to some extent as shown in Table 5, selecting the most weighed Factor of each component in the factor matrix, calcium, phosphorus, magnesium and manganese most weighed in the positive direction of F1, bilirubin and palmitate in the positive direction of F2, cholesterol in the negative direction of F2, and the residue, copper and zinc in the positive direction of F3. The three gallstone types were first divided into two groups: black stones and others on the F1 axis. Second, the others were subdivided into bilirubin stones and cholesterol stones on the F2 axis. Thus, cholesterol stones were found to consist of cholesterol, bilirubin stones of bilirubin and palmitate, and black stones of calcium, phosphorus, magnesium and manganese.

On the other hand, the residue, copper and zinc which weighed to F3, did not substantially contribute to the gallstone classification as the other components. The distribution of gallstones on the F3 axis showed that some of the bilirubin stones overlapped some of the black stones, and

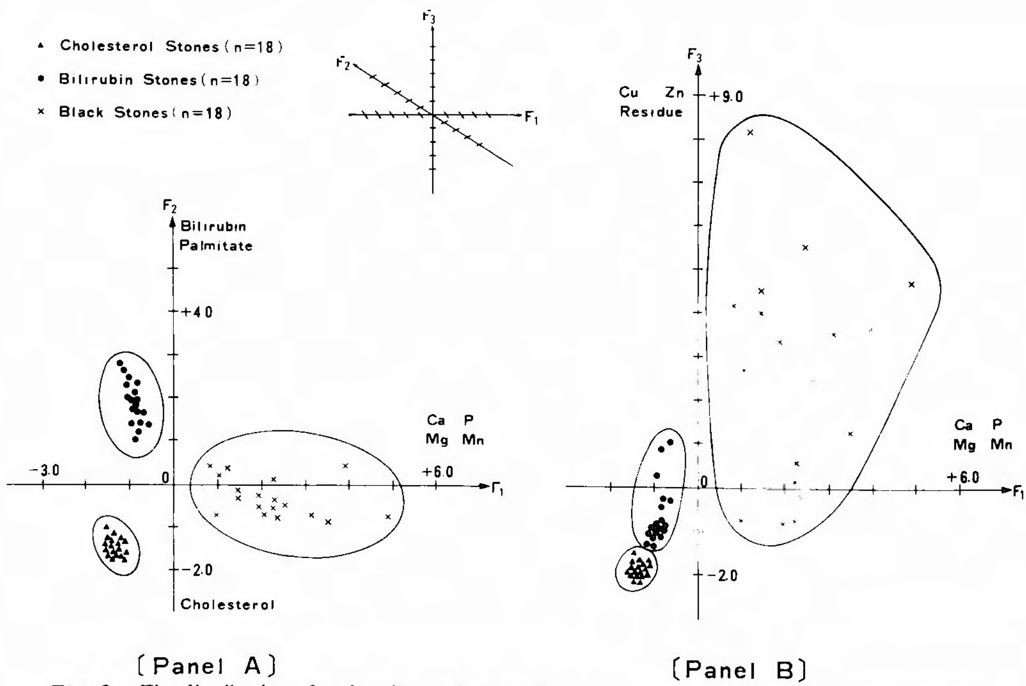

Fig. 2. The distribution of each gallstone in three-dimensional space determined by factor analysis.

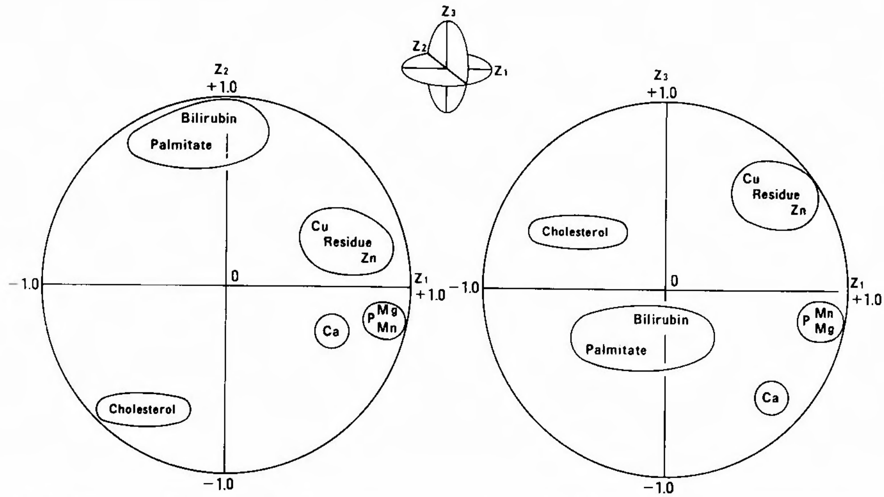

Fig. 3. The classification of gallstone components determined by principal component analysis.

the position of some bilirubin stones was adjacent to the cholesterol stones. Thus, these components must not be determinant factors in gallstone classification.

Classification of gallstone components: Though the relationship of gallstone components could be inferred also from correlation analysis, the results are too complex and obscure to be understood. In order to identify the relationship of them mathematically and more clearly, all gallstone components analysed here were plotted in the sphere (radius, 1.0) with Z1, Z2 and Z3 planes (Fig. 3), based on the factor loading of each gallstone component in principal component analysis shown in Table 6. Factor loading indicates the correlation between a given 'Component' (Zi) and a given variable (gallstone component) up to 1.0. Thus, the nearer the values of them are to 1.0, the more the variables are accounted for by a 'Component' (Zi)[23]. First, each gallstone component was plotted in two planes: Z1 and Z2, or Z1 and Z3. By combining these two planes

Table 6. Factor Loading of Each Component in 54 Gallstones Determined by Principal Component Analysis.

Component		Z_1	Z_2	Z_3
1.	Cholesterol	−.641	−.663	.314
2.	Bilirubin	−.049	.930	−.196
3.	Palmitate	−.297	.814	−.291
4.	Ca	.611	−.244	−.585
5.	P	.817	−.177	−.193
6.	Mg	.898	−.132	−.198
7.	Cu	.576	.322	.621
8.	Mn	.878	−.191	−.183
9.	Zn	.641	.160	.381
10.	Residue	.655	.236	.544
	Cummulative percentage	42.9	65.9	80.9(%)

using the sketch in the center, a sphere with three axes can be imaged. The nearer the position of each component is, the greater is their relationship.

From this figure, gallstone components may be classified into five groups: cholesterol, bilirubin and palmitate, copper, the residue and zinc, manganese, phosphorus and magnesium, and calcium. However, the position of each group is also significant; a single group and two complex groups with several components were found. Cholesterol belonged to the single group and this group is apparently apart from other groups. Therefore, cholesterol may be entirely independent on the other components in gallstones. Copper was in one of the complex groups with the residue and zinc. This evidence indicates clearly the close relationship between copper and the residue. On the other hand, manganese belonged to the other complex group with phosphorus and magnesium, which is located adjacent to calcium. These findings might be due to an association of manganese and magnesium with calcium phosphate as are apatite and whitlockite[4,5]. However, the position of calcium is complicated and not entirely the same as the manganese group. Perhaps, it is due to the multiple affinities of calcium with many substances such as carbonate, palmitate, phosphate and bilirubinate[4,31].

Intrahepatic Stones

In Japan, until now, intrahepatic stones were considered to be bilirubin stones and were thought to have the same origin. But, recently MUKAIHARA reported that not all of them were bilirubin stones; other stones containing more than 50% cholesterol were found in the stone type[21]. In this work, after analysing 10 components in 24 intrahepatic stones, confirmed to have originated in the intrahepatic duct, factor analysis was undertaken together with other stones. As shown in Table 2, cholesterol in these stones was significantly higher than that of bilirubin

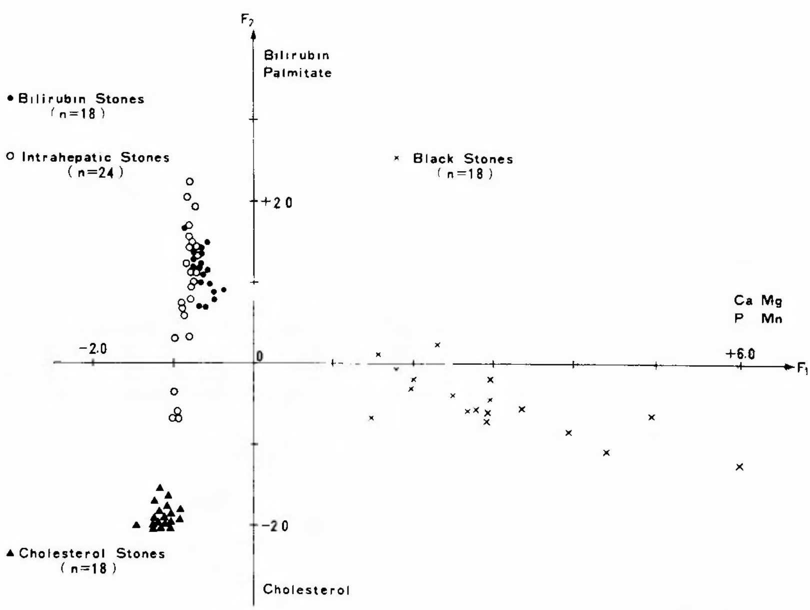

Fig. 4. The distributions of the intrahepatic stones and other gallstones.

stones in the choledochus; and phosphorus in these stones was lower ($P<0.005$). On the other hand, there was not a large difference among the other components between these two groups.

As seen in the depiction of 78 gallstones in the intrahepatic duct and other locations in two-dimensional space, most of the intrahepatic stones overlapped the bilirubin stones (Fig. 4); undoubtedly there were atypical intrahepatic stones, which differed from bilirubin stones and contained a large amount of cholesterol or palmitate.

II. Identification of Trace Elements in Gallstones.

Trace Elements vs Other Metals

Until now, there have been some reports concerning calcium, phosphate and carbonate with respect to some of trace elements using spectroscopy, however, as yet, no attempt has been made to quantify precisely the major ions and trace elements with respect to different types of gallstones. Thus, sodium, potassium and iron in addition to 6 metals described above (Table 2) were simultaneously determined in three types of gallstones (Table 7).

The amount of all the metals was the highest in black stones and the lowest in cholesterol stones. Among these 9 metals, calcium was overwhelmingly dominant in all types of gallstones, but the ranks of the others were not constant among the three types (Fig. 5). For example, copper was 7th next to potassium in cholesterol stones, but it was third in bilirubin stones, and in black stones it was fourth. On the other hand, manganese was 8th in both cholesterol and bilirubin stones, but in black stones, it was 6th next to copper. Thus, the ranks of metals were different according to the type of gallstone.

The concentrations of trace elements in the bile, although widely distributed and differing from those of the other ions[26], are considerably less than those ions in bile. On the contrary, some gallstones contained greater concentrations of copper and manganese than of some of the major ions. These discrepancies between bile and gallstones, also observed among the major ions, may suggest the importance of their state (free or bound) in bile, not the degree of their concentrations; the degree of ionization of the metal may be a significant factor for their presence in the stones.

Trace Elements vs Organic or Inorganic Compounds

To identify chemically the presence of copper and manganese in gallstones, their concentrations in the solutions during the extraction procedure and in the residue of six black stones

Table 7. The Concentrations of Other Metals in Various Gallstones.

Metal ($\mu g/g$)	Cholesterol Stones	Bilirubin Stones	Black Stones
Na	514 $\pm$62.1	4630$\pm$257	5080$\pm$660
Fe	51.6$\pm$22.2	382$\pm$142	683$\pm$122
K	42.7$\pm$14.8	331$\pm$ 57.8	598$\pm$102

(Mean$\pm$S.E.)

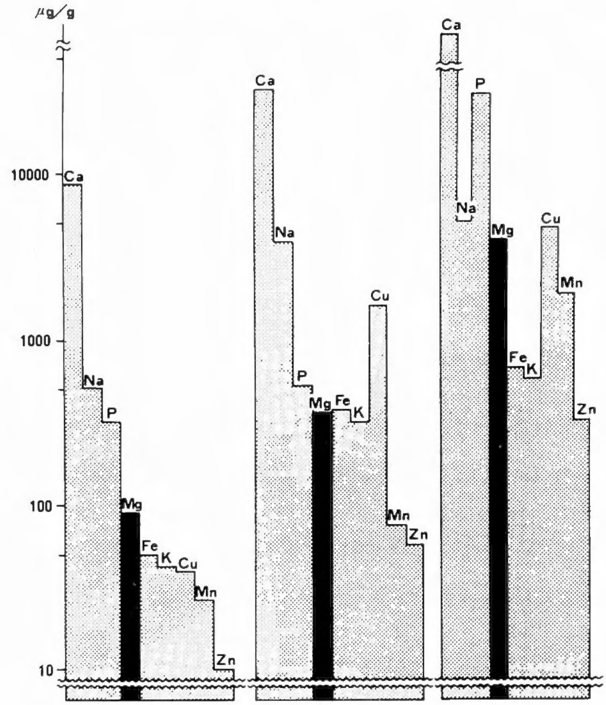

Fig. 5. The mean values of 9 metals in three types of gallstones.

and four bilirubin stones, were determined together with other metals. In the first and third fractions with petroleum ether, little metal was detected. Excluding this fraction, the metal extracted in each solution possibly originates from gallstones, as follows; the metal in the second fraction with 1 N-HCl was extracted from inorganic compounds or bounded metals to organic components, and the metal in the fourth fraction with DMSO was tightly bounded to organic substances or inorganic compounds.

In black stones (Table 8), a large proportion of manganese was present in the second fraction

Table 8. Metal Analysis of the Fractions during the Extraction Procedure of Black Stones.

Gallstones	Fraction	Cu (ng/ml)*	Mn (ng/ml)*	Fraction	Cu (ng/ml)*	Mn (ng/ml)*	Fraction	Cu (μg/g)**	Fraction	Cu (μg/g)**
1)	II	2830	1120	IV	2460	32.1	V	2090	VI	177
2)	II	8750	825	IV	2360	33.8	V	10600	VI	177
3)	II	3770	1500	IV	1740	8.0	V	1870	VI	134
4)	II	1970	1700	IV	645	11.5	V	1220	VI	190
5)	II	9320	884	IV	2240	29.1	V	2250	VI	98
6)	II	1090	47	IV	777	16.0	V	1890	VI	170

I. petroleum ether, II, 1N-HCl, III. petroleum ether, IV. dimethyl sulfoxide, V. residue, VI. final residue
*: the concentration of each solution.
**: the concentration in the residue or the final residue.

Table 9. Metal Analysis of the Fractions during the Extraction Procedure of Bilirubin Stones.

Gallstones	Fraction	Cu (ng/ml)	Fraction	Cu (ng/ml)	Fraction	Cu (μg/g)
1)	II	1930	IV	2900	V	7000
2)	II	2690	IV	1770	V	4450
3)	II	165	IV	542	V	398
4)	II	260	IV	185	V	228

II, IV, V: see Table 8.

and a small amount in the fourth fraction, as with calcium and other. On the contrary, copper was present in both the second and fourth fractions, and importantly, also was present, to a lesser degree in the residue. Their mean ratios to total copper in six gallstones were as follows: 60% for 1 N-HCl solution, 27.3% for DMSO solution and 12.7% in the residue. This residue had too little metal (such as calcium, magnesium, phosphorus, iron and manganese) to be very significant. Thus, manganese was mainly seen in the 1 N-HCl solution, possibly due to an inorganic compound such as calcium phosphate[4,5]. On the other hand, copper was not easily released by 1 N-HCl and may be present in organic substances such as bilirubin dissolved in DMSO. Furthermore, copper is the sole metal in the residue among the metals analysed here.

Also in bilirubin stones (Table 9), copper was present in the fraction of 1N-HCl and DMSO and also in the residue, which contained no other metals.

These results directly confirmed the close relationship between trace elements and other components of gallstones by statistical analysis (Fig. 3).

Purification and Identification of the Residue

The residues of six black stones were further analysed. First, 6 N-NaOH was added to the residue and heated for 72 hours at 110°C. Because of this procedure, the residue was dissolved completely into a black brown solution, but when neutralized by 6 N-HCl, a black substance (final residue) was formed in the solution with white flocculations which might have come from the glassware. The proportion of the 'final residue' to the residue before this treatment was 8.3% to 37% in six cases. When the copper content of the 'final residue' was determined by the wet digestion method, of much importance, its concentration (97.6 to 190 μg/g) was in a fairly narrow range compared to the value of the other fractions (Table 8). This evidence strongly indicates that the 'final residue' may be the most purified substance in the residue, and it may be defined as an organic compound containing about 170 μg/g of copper.

On the other hand, a considerable amount of various amino acids were detected in the supernatant of the neutralized solution, and whether the amino acids originated from protein, peptides, protein binding substance or amino acids in gallstones was unknown.

Thus, the residue was defined as a complex compound consisting of at least two kinds of copper binding organic substances such as a protein-like substance and final residue, which have not yet been identified.

Discussion

Although the composition of gallstones is highly complicated due to the fact that they consist of various components, the general configurations of them can be more clearly identified because of the development of both analytical techniques for gallstone components and data management.

Figure 2 which presents the major findings of this work derived from chemical and statistical analysis, provides much information with regard to not only gallstone classification but also its pathogenesis.

At present, there is much confusion concerning the classification of gallstones because the same components are present, more or less, in all gallstones. For example, cholesterol stones for the most part consist mostly of cholesterol, but bilirubin and other components are also present though their proportions are smaller. Thus, it may be advantageous to classify the stones according to the proportion of their components, instead of specific components since, so-called cholesterol stones do not consist of cholesterol alone, and bilirubin stones do not consist of only bilirubin. Therefore, as shown in Fig. 2, cholesterol stones are defined as those mainly consisting of cholesterol, likewise, bilirubin stones consist mainly of bilirubin and palmitate, and black stones mainly consist of metals such as calcium, magnesium, phosphorus and manganese. From visual classification bilirubin stones and black stones have sometimes been considered as being in the same group, but from component analysis, a distinction should be made between these two types. The components not shown in panel A of Fig. 2 such as the residue, copper, and zinc, contribute very little to the classification. The residue is black and the highest concentration of it is seen in black stones, but it is also present in bilirubin stones to a considerable degree. This finding, along with the evidence that a bilirubin component is also present in black stones, may be the chief reason for the confusion in visual classification between bilirubin stones and black stones.

The aim of this work is to define and clarify the most important components in the formation of each type of gallstone and the role of trace elements in its process. Although both copper and manganese are among the trace elements mainly excreted into bile, their excretion mechanism and state in the bile are quite different[26]. While free copper has not been detected and most of the copper in bile was considered to be bounded to some carrier (for its toxicity) which has not yet been identified[2,15,19,26]. On the other hand, free manganese exists in the bile together with bounded Mn[36,37]; as in case of calcium[29,30] and other metals. This evidence may be the chief reason why manganese and other metals are present in the gallstones, and their origin in the stones may be free metals in the bile. Also, cholesterol and bilirubin in gallstones may originate from supersaturated cholesterol and unconjugated bilirubin in bile, both of which are hardly soluble in the bile.

Admirand and Small have found insoluble cholesterol due to the supersaturation in pathologic bile with cholesterol stones, but they emphasized that this finding was only one step in the formation of the stones and other factors were necessary in the process from the microcrystalline to macroscopic cholesterol gallstones[1]. Holzbach modified the micellar zone indicated by

ADMIRAND and SMALL[17], he reported also that cholesterol supersaturation of bile alone is not a sufficient cause for gallstone formation, because supersaturation was seen in not only pathologic bile but also normal human bile. Thus, while cholesterol is a major component in cholesterol stones, pigment seen in the center of them may influence the subsequent precipitation of cholesterol as described by BOGREN and BEEN[5,6].

Bilirubin stones are common in Japan and according to MAKI[18] the major process and cause of these stones are the coagulation of unconjugated bilirubin with calcium, originating from hydrolysis of conjugated bilirubin in bile by the β-glucuronidase of E. coli due to infection and stasis, and solidification of calcium bilirubinate by the bridging action of a high molecular weight substance. This theory is significant to classify logically the process to the solid concrement from bile components and to differentiate the precipitates or particles in bile from the concrement. However, in bilirubin stones, a considerable amount of palmitate and cholesterol as well as bilirubin and calcium are present, and this cannot be adequately explained. Furthermore, it is obscure whether the infection and stasis of bile is the causal events before the gallstone formation or the secondary events resulting from the presences of the stones. From recent analysis, unconjugated bilirubin other than conjugated bilirubin is also present, though a small amount, in hepatic bile or gallbladder bile with no infection[3,7,11,28,38]. In addition, the mere presence of unconjugated bilirubin in bile may not induce the formation of gallstone, and neither may the presence of supersaturated cholesterol induce cholesterol stones.

Though the origin of the residue is unknown, it is an important organic substance in black stones. As described above, the residue consisted of a protein-like substance and an unknown substance containing copper (final residue). WOSIEWITZ reported from the chemical analysis that black stones contained a large amount of low molecular and high molecular bile pigment polymers (29.8% and 55.5%, respectively). This high molecular bile pigment, which was also called 'black pigment', may be similar to the residue in this study, and likewise, to the 'black pigment' reported by BURNETT or SUZUKI[10,33,34,35,40]. BURNETT described that in addition to copper, calcium and sulphur were present in 'black pigment', and calcium might be the most important metal involved in the formation of the pigment. However, the residue in the present study contained little or no calcium. This difference may be due to the extraction procedure sequence; namely, the order in which HCl and DMSO are used. The tightly bounded metal complex was not easily dissolved in DMSO without HCl, and tightly bounded metals was not completely released in a relative low valent HCl. In addition, metal analysis was carried out in the 'black pigment' according to BURNETT's method and also by reversing the order of HCl and DMSO as in MUKAIHARA's method, using the same gallstone powder of black stone. In them with both of these methods, calcium as well as copper were detected. Thus, copper is the most important metal because it is present in both the 'black pigment' and the residue.

A black copper containing substance, similar to the residue, was seen in the microscopic study of the so-called cholesterol stones. According to BEEN[4,5], some copper was present in the black sulphur band of pigment layers which had the character of a protein-pigment complex. Also, the residue contained sulphur which might have been involved in amino acids, but in the 'final residue' it was rarely detected by fluorescent X ray. This 'final residue' in the present study, refined by using NaOH and HCl, may be the precipitation formed from 'black pigment' by a similar treatment, observed by WOSIEWITZ. Thus, while there is no clear evidence, the 'final residue' appears to be the high polymer originating from bilirubin as described by WOSIEWITZ and others. Due to the high incidence of black stones in liver cirrhosis[9,28,39], hemolytic anemia[12,28] and WILSON's disease[25], the residue or the 'final residue' may originate from metabolic disorder or excretion disorder in the liver.

While the role of various gallstone components in their formation is highly complicated, Figure 2 may shed light on the solution of this complex problem. The character and presence of these components in bile and gallstones shown in the F3 axis of panel B are quite different from those in panel A. The residue or the 'final residue' which is a copper containing substance may play the most important role in gallstone formation, behaving as a resin and it may promote gallstone formation due to the adsorption with highly insoluble bile components such as supersaturated cholesterol, bilirubin-metal complex and calcium containing compounds in conjunction with other events such as inflammation, stasis and the injury of biliary tracts[3,8,24]. Therefore, the components shown in panel A of Fig. 2 may be secondary materials as a result of the adsorption, while they are major components in each type of gallstones.

Conclusions

Trace elements were determined together with cholesterol, bilirubin and other metals in 84 gallstones; cholesterol stones, bilirubin stones, black stones and intrahepatic stones extirpated intraoperatively.

1) The highest concentrations of trace elements, as well as other metals, were seen in black stones, and the lowest in cholesterol stones. Especially, in black stones, the amount of manganese was remarkable.

2) From the statistical analysis, copper was highly correlated to the black residue after extraction procedure, and manganese was related to phosphorus. Gallstones were classified according to macroscopic classification due to their components from factor analysis. However, the residue and copper were not determinant components in gallstone classification.

3) It was verified from the factor analysis that intrahepatic stones involve atypical stones containing a large amount of cholesterol or palmitate which can not be considered as bilirubin stones.

4) Black stones contained the highest proportions of all metals including sodium, potassium and iron. However, the rank of metal concentration was varied not only among the gallstone types but also from that in the bile.

5) Most of the manganese was present in the 1N-HCl solution of the extraction procedure of gallstones, but copper was seen in the DMSO solution and the residue, not only in the 1N-HCl solution.

6) The residue consisted of a protein-like substance and an unknown copper-binding substance (final residue).

From these results, gallstones were classified into three groups; cholesterol stones, bilirubin stones and black stones. A copper-binding substance (residue or final residue) may be the most important component in gallstone formation.

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和文抄録

胆石形成における微量元素の役割に関する化学的・統計学的研究

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手術的に摘出した200個のヒト胆石より，肉眼的に最も典型的なコレステロール石，ビリルビン石，黒色石を18個づつ選出し，銅，マンガン，亜鉛といった微量元素を測定すると同時に，コレステロール，ビリルビン，脂肪酸，カルシウム，マグネシウム，リン，さらに胆石抽出操作後にも残る黒色成分（残渣）をも測定し，コンピューターを用いて多変量解析を行い，数学的，統計学的に各胆石の成分的特徴を明らかにすると共に，胆石中における微量元素の役割について検討を加え，以下の結果を得た．

1) いずれの微量元素も，その含有量は黒色石に最も多く，コレステロール石に最も少なかった．特にマンガンは，黒色石に極めて多く含まれているのが特徴的であった．ただし，銅はビリルビン石にもかなり含有されていた．

2) 一方，因子分析により，各胆石を Factor score でもって3次元空間に plot すると，54個の胆石は肉眼的分類と一致して3群に分類され，コレステロール石はコレステロール，ビリルビン石はビリルビンと脂肪酸，黒色石はカルシウム，マグネシウム，リン，マンガンを多く含む石であると立証された．銅，残渣成分は，各胆石群で一部重複し，他成分程胆石分類に関与していなかった．

また，主成分分析にて各胆石成分相互の関係を検討すると，銅は残渣成分と，マンガンはリン，マグネシウム，及びカルシウムと密接な関係を有することが明らかとなった．

3) さらに，24個の肝内結石を同時に分析して因子分析を行うと，肝内結石は大部分ビリルビン石に属するものの，コレステロール石に近い石や脂肪酸を多量に含有する石も認められ，化学分析，統計分析により，肝内結石は必ずしもビリルビン石のみではないと実証し得た．

4) 有機物を除く上記の6元素の他に，胆石中のナトリウム，カリウム，鉄をも同時に測定し，各胆石群におけるこれら9元素の平均含有量を比較すると，銅はコレステロール石では7番目，ビリルビン石では3番目，黒色石では4番目に多かった．微量元素は，胆汁中では他の主要電解質イオンより明らかに低濃度であるにも拘らず，胆石中では微量元素の方が多い石もあり，胆石形成における微量元素の重要な関与が示唆された．

5) 胆石中の有機物抽出に用いた溶媒中の微量元素を分析した結果，大部分のマンガンはカルシウム等と同様1N-HCl溶液に抽出されていたが，銅はDMSO溶液中や残渣成分中にもかなり含有されていた．この残渣成分には，銅以外の金属は検出できなかった．

6) さらに，6個の黒色石中の残渣成分を，アルカリ加水分解で溶解後，強塩酸で中和すると，アミノ酸を含む溶液と黒い沈殿物（最終残渣）が形成された．この最終残渣は銅を一定濃度で含有していた．

以上の結果より，ヒト胆石はその化学的，統計学的分析により3群の胆石に分別できることが実証された．その中の黒色石について詳細に分析した結果，Copper-binding substance である残渣成分，あるいは最終残渣成分が，最も重要な構成成分であると推測され，胆石形成に際し，銅が重要な役割をなしている可能性が強く示唆された．